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(54) Title: FATTY-ACID AMIDE HYDROLASE

(57) Abstract

The soporific activity of *cis*-9,10-octadecenoamide and other soporific fatty-acid primary amides is neutralized by hydrolysis in the presence of fatty-acid amide hydrolase (FAAH). Hydrolysis of *cis*-9;10-octadecenoamide by FAAH leads to the formation of oleic acid, a compound without soporific activity. FAAH has been isolated and the gene encoding FAAH has been cloned, sequenced, and used to express recombinant FAAH. Inhibitors of FAAH are disclosed to block the hydrolase activity.

FATTY-ACID AMIDE HYDROLASE

DESCRIPTION

Technical

The invention relates to an enzyme which catalyzes a hydrolytic conversion between soporific fatty acid primary amides and their corresponding fatty acids and is designated a fatty-acid amide hydrolase (FAAH), to methods for enzymatically catalyzing such conversions, and to methods for inhibiting the enzymatic catalysis of such conversions. More particularly, the invention relates to FAAH protein, in either isolated or recombinant form, and to its use and inhibition.

Statement of Government Rights

This invention was made with government support under a National Institutes of Health Shared Instrumentation grant No. 1 S10 RR07273-01. The government has certain rights in the invention.

Background

Sleep is a natural, periodic behavioral state during which the body rests itself and its physiological powers are restored. It is characterized by a loss of reactivity to the environment. During sleep, certain physiological processes of both the body and the brain function differently than they do during alert wakefulness. Normal sleep consists of at least two quite different behavioral states: synchronized sleep, during which the electroencephalogram consists of slow waves of high amplitude, and desynchronized sleep (DS) or activated sleep characterized by rapid eye movements (REM sleep), in which the electroencephalogram pattern is characterized by waves of high frequency and low amplitude. Synchronized sleep is further characterized by slow and regular respiration, by relatively

constant heart rate and blood pressure, and by a predominance of delta waves. Synchronized sleep usually consists of four stages, followed by a period of activated sleep. Each cycle lasts between 80 and 120 minutes. In contrast, desynchronized sleep is further characterized by irregular heart rate and respiration, periods of involuntary muscular jerks and movements, and a higher threshold for arousal. Periods of desynchronized sleep last from 5-20 minutes and occur at about 90 minute intervals during a normal night's sleep.

Sleep disorders include sleep deprivation and paroxysmal sleep, i.e., narcolepsy. There has been no known pharmacological method for promoting or inhibiting the initiation of sleep or for maintaining the sleeping or waking state.

Cerebrospinal fluid (liquor cerebrosinalis) is a clear, colorless fluid that circulates within the four ventricles of the brain and the subarachnoid spaces surrounding the brain and spinal cord. Cerebrospinal fluid originates as an ultrafiltrate of the blood secreted by the choroid plexus in the lateral third and fourth ventricles. Cerebrospinal fluid is also sometimes called neurolymph. After passing through the four ventricles and the subarachnoid spaces, cerebrospinal fluid is largely resorbed into the venous system via the arachnoid villi. Cerebrospinal fluid serves as a medium for the removal of catabolites, excretions, and waste materials from the tissues bathed by it. To date, no factor derived from cerebrospinal fluid has been reported to correlate with sleep deprivation. What is needed is a method for analyzing cerebrospinal fluid for identifying a biochemical factor generated by subject that correlates with sleep deprivation.

Since the seminal discovery of prostaglandins, there has

been increasing recognition of the role of fatty acids and their derivatives in important physiological processes, e.g., B. Samuelsson, Les Prix Nobel 1982, pp. 153-174.

Cis-9,10-Octadecenoamide has been isolated from the cerebrospinal fluid of sleep-deprived cats and has been shown to exhibit sleep-inducing properties when injected into rats. Other fatty acid primary amides in addition to *cis*-9,10-octadecenoamide were identified as natural constituents of the cerebrospinal fluid of cat, rat, and man, indicating that these compounds compose a distinct family of brain lipids. Together, these results teach that fatty acid primary amides represent a new class of biological signalling molecules that can be employed for inducing subjects to sleep. Preferred fatty acid primary amides include an alkyl chain having an unsaturation and are represented by the following formula: $NH_2C(O)(CH_2)_{(6\geq n\leq 11)}CH{=}CH(CH_2)_{(8\geq n\leq 5)}CH_3$. Preferred soporific fatty acid primary amides have an unsaturation with a *cis* configuration within their alkyl chain. In addition to *cis*-9,10-octadecenoamide, other soporifically active fatty acid primary amides include *cis*-8,9-octadecenoamide, *cis*-11,12-octadecenoamide, and *cis*-13,14- docosenoamide.

Deutsch et al, Biochem. Pharmacol.,46:791 (1993) has identified an amidase activity which catalyzes both the hydrolysis and synthesis of arachidonylethanolamide (anandamide) from the membrane subcellular fractions taken from neuroblastoma, glioma cells and crude homogenates of rat brain tissues. The study detected the uptake and enzymatic breakdown of arachidonylethanolamide (anandamide) to arachidonic acid (and *vice versa*) from the homogenates of tissues from brain, liver, kidney and lung but not from rat heart and skeletal muscles.

The active membrane fraction which displayed this amidase

activity was prepared by either homogenizing the desired cell line and subsequently subjecting the crude homogenate to density centrifugation or by taking the crude homogenates of rat brains and directly incubating them with anandamide.

The uptake and degradation of arachidonylethanolamide (anandamide) was assayed by incubation of [^{3}H]-anandamide (NEN, NET-1073, 210 Ci/mmol) in the cell culture medium. It was found, by liquid scintillation counting of the aqueous and organic phases, that arachidonic acid and anandamide distributed in the organic phase. Thus, the organic extract of the cell medium was subsequently visualized using thin-layer chromatography, sprayed with a surface autoradiograph enhancer (EN^{3}HANCE, Dupont) and exposed to X-ray film (Kodak X-OMAT AR) at -80 °C.

The serine protease inhibitor, phenylmethylsulfonyl fluoride at 1.5 mM concentration completely inhibited the amidase activity. Other inhibitors tested had little or no effect on the activity and included aprotinin, benzamidine, leupeptin, chymostatin and pepstatin.

In a second manuscript, Deusch et. al..(*J. Biol Chem.*, **1994**, *269*, 22937) reports the synthesis of several types of specific inhibitors of anandamide hydrolysis and their ability to inhibit anandamide breakdown *in vitro*. Four classes of compounds were synthesized and include fatty acyl ethanolamides, α-keto ethanolamides, α-keto ethyl esters and trifluoromethyl ketones. The most effective class of compounds were the trifluoromethyl ketones and α-keto esters. The least potent inhibitors were the α-keto amides and saturated analogs of anandamide.

As an example, when anandamide is incubated with neuroblastoma cells, it is rapidly hydrolyzed to arachidonate

but in the presence of the inhibitor arachidonyl trifluoromethyl ketone, there is a 5 fold increase of anandamide levels. The study infers that polar carbonyls such as those found in trifluoromethyl ketones, may form stabilized hydrates that mimic the tetrahedral intermediates formed during the reaction between the nucleophilic residue and the carbonyl group of anandamide. Deutsch suggests that the nucleophilic residue may be the active site of a serine hydroxyl in the hydrolytic enzyme.

This enzyme is classified as an amidase (EC #3.5) where the enzyme acts on carbon nitrogen bonds other than peptide bonds. The amidase activity is inhibited by the serine protease inhibitor, PMSF and the action of trifluoromethyl ketone inhibitors (and others) directly affect the hydrolytic activity of the enzyme. Furthermore, Deutsch suggests that anandamide is cleaved by a mechanism that involves an active site serine hydroxyl group.

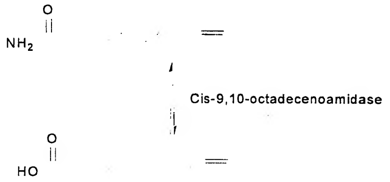
What is needed is an identification of enzymes within the brain tissue which catalyze the degradation of soporific compound found in the cerebrospinal, for mediating the soporific activity of these compounds. What is needed is an identification of inhibitors for inhibiting the activity of enzymes which degrade soporific compounds of the type found in cerebrospinal fluid.

Brief Summary of the Invention

An enzyme is disclosed herein which degrades soporific fatty acid primary amides, and is designated fatty-acid amide hydrolase, or FAAH. FAAH is one of the enzymes which mediates the activity of fatty acid primary amides, including soporific fatty acid primary amides.

As disclosed herein, FAAH is characterized by an enzymic

activity for catalyzing a conversion *cis*-9,10-octadecenoamide to oleic acid, among other substrates, as shown in Scheme 1 below, and therefor was originally identified as *cis*-9,10-octadecenoamidase. However, it is now shown that FAAH has activity to hydrolyse a variety of fatty acid primary amides, and therefore the amidase originally referred to as *cis*-9,10-octadecenoamidase is more appropriately referred to as FAAH.



SCHEME 1

One aspect of the invention is directed to a purified form of FAAH. FAAH can be purified by a variety of methods, including a chromatographic methodology. Preferred chromatographic methodologies include affinity chromatography, electric chromatography, gel filtration chromatography, ion exchange chromatography, and partition chromatography. In affinity chromatography, a solid phase adsorbent contains groups that bind particular proteins because they resemble ligands for

which the proteins have a natural affinity. In a preferred mode, the solid phase adsorbent contains one or more FAAH inhibitors which bind the enzyme. In antibody affinity chromatography, a solid phase immunoabsorbent having antibodies with a bind specificity with respect to FAAH are employed. In electric chromatography or electrophoresis, the FAAH is separated from other molecules according to its molecular weight or isoelectric point. In gel filtration, also known as gel permeation, molecular sieve, and exclusion chromatography, the solid phase creates a stationary phase of gel solvent and a mobile phase of excluded solvent. The FAAH is separated according to its molecular size as it partitions between the stationary and mobile phases. The gel particles are selected to have a exclusion size in excess of FAAH. In ion exchange chromatography, a solid phase ion exchanger is employed for separating the FAAH from other molecules according to its partitioning between ionic and nonionic forces. In partition chromatography, immiscible fluids having a stationary and mobile phases are employed for separating the FAAH according to its partitioning between the two immiscible phases. Preferred chromatographic methodologies include DEAE chromatography, affinity chromatography on a solid phase having attached Hg groups derivatized with an inhibitor of FAAH such as a trifluoroketone.

In a preferred mode, a crude source of FAAH is purified in four steps. In the first step, a crude source of FAAH is purified by exchange chromatography using a DEAE chromatography column to form a first elution product. In the second step, the elution product from the first step is further purified by partitioning by with affinity chromatography to form a second elution product. In the third step, elution product from the

second step is further purified by partitioning with Heparin affinity chromatography to form a third elution product. In the fourth step, the elution product from the third step is further purified by partitioning with an stationary phase derivatized with a trifluoroketone inhibitor of FAAH. The eluant from the fourth step form the purified form of FAAH.

FAAH can be isolated from any of a variety of mammalian species, including rat, mouse or human, as described herein.

Fatty-acid amid hydrolase (FAAH) is characterized by inclusion of an amino acid sequence selected from a group consisting of:

a.) GGSSGGEGALIGSGGSPLGLGTDIGGSIRFP (**SEQ ID NO 5**),

b.) SPGGSSGGEGALIGS (**SEQ ID NO 6**),

c.) ALIGSGGSPLGLGTD (**SEQ ID NO 7**),

d.) GLGTDIGGSIRFPSA (**SEQ ID NO 8**),

e.) RFPSAFCGICGLKPT (**SEQ ID NO 9**),

f.) GLKPTGNRLSKSGLK (**SEQ ID NO 10**),

g.) KSGLKGCVYGQTAVQ (**SEQ ID NO 11**),

h.) QTAVQLSLGPMARDV (**SEQ ID NO 12**),

i.) MARDVESLALCLKAL (**SEQ ID NO 13**),

j.) CLKALLCEHLFTLDP (**SEQ ID NO 14**),

k.) FTLDPTVPPFPFREE (**SEQ ID NO 15**),

l.) PFREEVYRSSRPLRV (**SEQ ID NO 16**),

m.) RPLRVGYYETDNYTM (**SEQ ID NO 17**),

n.) DNYTMPSPAMRRALI (**SEQ ID NO 18**),

o.) RRALIETKQRLEAAG (**SEQ ID NO 19**),

p.) LEAAGHTLIPFLPNN (**SEQ ID NO 20**),

q.) FLPNNIPYALEVLSA (**SEQ ID NO 21**),

r.) EVLSAGGLFSDGGRS (**SEQ ID NO 22**),

s.) DGGRSFLQNFKGDFV (**SEQ ID NO 23**),

t.) KGDFVDPCLGDLILI (**SEQ ID NO 24**),

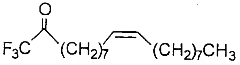
u.) DLILILRLPSWFKRL (**SEQ ID NO 25**),
v.) WFKRLLSLLLKPLFP (**SEQ ID NO 26**),
w.) KPLFPRLAAFLNSMR (**SEQ ID NO 27**),
x.) LNSMRPRSAEKLWKL (**SEQ ID NO 28**),
y.) KLWKLQHEIEMYRQS (**SEQ ID NO 29**),
z.) MYRQSVIAQWKAMNL (**SEQ ID NO 30**),
aa.) KAMNLDVLLTPMLGP (**SEQ ID NO 31**), and
ab.) PMLGPALDLNTPGR (**SEQ ID NO 32**).

Another aspect of the invention is directed to a method for catalyzing the hydrolysis of a fatty acid primary amide. In this hydrolysis method, the fatty acid primary amide is combined or contacted with a catalytic amount of purified form of FAAH. In a preferred mode, the fatty acid primary amide is of a type which includes an alkyl chain having an unsaturation or more particularly is represented by the following formula:

$$NH_2C(O)(CH_2)_{(6\geq n\leq 11)}CH{=}CH(CH_2)_{(8\geq n\leq 5)}CH_3.$$

More particularly, the unsaturation of the alkyl chain may have a *cis* configuration or may be identically *cis*-9,10-octadecenoamide, *cis*-8,9-octadecenoamide, *cis*-11,12-octadecenoamide, or *cis*-13,14- docosenoamide.

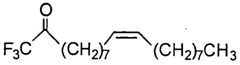
Another aspect of the invention is directed to a method for inhibiting an enzymatically catalyzed hydrolysis of fatty acid primary amides, such as *cis*-9,10-octadecenoamide, by FAAH. In this method, FAAH is combined or contacted with an inhibitor of FAAH. Preferred inhibitors include phenylmethylsulfonyl fluoride, $HgCl_2$, and a trifluoroketone having the following structure:



Another aspect of the invention is directed to a method for ascertaining the inhibitory activity of a candidate inhibitor of FAAH. Thus, FAAH is admixed with a candidate FAAH inhibitor to assess inhibitory capacity in a screening method.

In a preferred method for determining inhibitory activity of a candidate FAAH inhibitor, the contemplated method comprises five steps. In the first step, a mixture "A" is formed by combining FAAH and *cis*-9,10-octadecenoamide substrate under reaction conditions. In the second step, a mixture "B" is formed by combining the mixture "A" with the candidate inhibitor. In the third step, the conversion of *cis*-9,10-octadecenoamide substrate to a hydrolysis product within mixture "A" is quantified. In the fourth step, the conversion of *cis*-9,10-octadecenoamide substrate to hydrolysis product within mixture "B" is quantified. In the fifth step, the inhibitory activity of the candidate inhibitor is ascertained by comparing the quantifications of steps three and four.

Another aspect of the invention is directed to a trifluoroketone inhibitor of FAAH represented by following structure:



Another aspect of the invention is directed to one or more nucleotide sequences the encode part or all of FAAH. The complete nucleotide sequence that encodes human, mouse or rat

FAAH are shown in SEQ ID Nos. 42, 39 or 35, respectively.

The partial nucleotide sequence of rat FAAH is represented as follows:

CCAGGAGGTTCCTCAGGGGGTGAGGGGGCTCTCATTGGATCTGGAGGTTCCCCT
CTGGGTTTAGGCACTGACATTGGCGGCAGCATCCGGTTCCCTTCTGCCTTCTGC
GGCATCTGTGGCCTCAAGCCTACTGGCAACCGCCTCAGCAAGAGTGGCCTGAAG
GGCTGTGTCTATGGACAGACGGCAGTGCAGCTTTCTCTTGGCCCCATGGCCCGG
GATGTGGAGAGCCTGGCGCTATGCCTGAAAGCTCTACTGTGTGAGCACTTGTTC
ACCTTGGACCCTACCGTGCCTCCCTTTCCCTTCAGAGAGGAGGTCTATAGAAGT
TCTAGACCCCTGCGTGTGGGGTACTATGAGACTGACAACTATACCATGCCCAGC
CCAGCTATGAGGAGGGCTCTGATAGAGACCAAGCAGAGACTTGAGGCTGCTGGC
CACACGCTGATTCCCTTCTTACCCAACAACATACCCTACGCCCTGGAGGTCCTG
TCTGCGGGCGGCCTGTTCAGTGACGGTGGCCGCAGTTTTCTCCAAAACTTCAAA
GGTGACTTTGTGGATCCCTGCTTGGGAGACCTGATCTTAATTCTGAGGCTGCCC
AGCTGGTTTAAAAGACTGCTGAGCCTCCTGCTGAAGCCTCTGTTTCCTCGGCTG
GCAGCCTTTCTCAACAGTATGCGTCCTCGGTCAGCTGAAAAGCTGTGGAAACTG
CAGCATGAGATTGAGATGTATCGCCAGTCTGTGATTGCCCAGTGGAAAGCGATG
AACTTGGATGTGCTGCTGACCCCNATGYTNGGNCCNGCNYTNGAYYTNAAYACN
CCNGGNMGN (**SEQ ID NO 54**).

Brief Description of the Drawings

Figure 1 illustrates the structures of natural agent, *cis*-9,10-octadecenoamide (**1**), related analogs (**2-6**). Compound **6** is the preferred structure for naturally occurring C_{22} fatty acid amide.

Figure 2 illustrates the determined partial amino acid sequence of the rat FAAH as described in Section B.4.

Figure 3 illustrates a partial purification strategy involving isolation of a plasma membrane protein fraction from rat liver using 1) a sucrose gradient of the liver membrane

followed by 2) a 100 mM sodium carbonate wash and 3) solubilization in trion-based buffer. The isolated liver plasma membrane is then purified by four consecutive chromatographic steps: 1) Ion exchane DEAE column, 2) Mercury inhibition column, 3) detergent exchange Heparin column followed by 4) an affinity column with a trifluoroketone inhibitor. The purified protein was determined to have a 20-30 fold enrichment of amidase activity from crude membrane protein fraction by visual comparison of the purified protein band intensity with the crude protein fraction. Estimated purified yield is 10-15% from crude liver plasma membrane protein.

Figure 4 illustrates the affinity column purification strategy (step 4, Figure 3) using a trifluoroketone inhibitor which is linked to a disulfide-derivatized solid support (pyridyl disulfide beads).

Figure 5 illustrates the synthetic protocol for the synthesis of the trifluoroketone inhibitor and subsequent attachment of the inhibitor to the disulfide-derivatized solid support using pyridyl disulfide beads.

Figure 6 represents an autoradiogram of a thin layer chromatography plate ($SiO2$, 55% ethyl acetate/hexanes) illustrating the FAAH activity of a rat brain membrane fraction with respect to the hydrolysis of radio-labelled *cis*-9,10-octadecenoamide to oleic acid and its inhibition by phenylmethyl sulfonyl fluoride (PMSF). Lane number, content: lane 1, *Cis*-9,10-octadecenoamide standard; lane 2, *Cis*-9,10-octadecenoamide with rat brain soluble fraction; lane 3, *Cis*-9,10-octadecenoamide with rat brain membrane fraction; lane 4, *Cis*-9,10-octadecenoamide with rat brain membrane fraction + 1 mM phenylmethylsulfonyl fluoride (PMSF); lane 5, *Cis*-9,10-octadecenoamide with rat brain membrane fraction + 5 mM EDTA;

lane 6, *Cis*-9,10-octadecenoamide with rat pancreatic microsomes; lane 7, *Cis*-9,10-octadecenoamide with proteinase K (200 mg); lane 8, oleic acid standard.

Figure 7 represents an autoradiogram of a thin layer chromatography plate (SiO2, 55% ethyl acetate/hexanes) illustrating the FAAH activity of a rat brain membrane fraction with respect to the hydrolysis of radio-labelled *cis*-9,10-octadecenoamide to oleic acid and its inhibition by mercuric chloride ($HgCl_2$). The optimal concentrations required for inhibition of amide hydrolysis activity lies between 50 mM and 5 mM $HgCl_2$. The various lanes of the TLC plate are identified as follows: lane 1, *Cis*-9,10-octadecenoamide standard; lane 2, *Cis*-9,10-octadecenoamide with rat brain soluble fraction; lane 3, *Cis*-9,10-octadecenoamide with rat brain membrane fraction and 500 mM $HgCl_2$; lane 4, *Cis*-9,10-octadecenoamide with rat brain membrane fraction and 50 mM $HgCl_2$; lane 5, *Cis*-9,10-octadecenoamide with rat brain membrane fraction and 5 mM $HgCl_2$; lane 6, oleic acid standard. A typical $HgCl_2$ inhibition study uses a 100 mM $HgCl_2$ stock (27 mg in 1mL Tris buffer (50 mM), pH 7.5) of $HgCl_2$.

Figure 8 represents a northern blot of mRNA obtained from cloning procedures. Ribosomal markers are shown by the arrows, next to lane 1, and indicate 5kb and 2kb bands. The arrow next to lane 6 points to a 3kb band which is representative of the oleic amidase enzyme. Lane 1 is total RNA from rat brain; lane 2 is total RNA from rat lung; lane 3 is total RNA from rat kidney; lane 4 is total RNA from rat heart; lane 5 is total RNA from rat liver; lane 6 is mRNA from rat liver (mRNA loaded in lane 6 is approximately 500 ng); total respective RNA loaded in lanes 1-5 was approximately 15 μg.

Figure 9 illustrates the deduced encoded amino acid residue

sequence of rat oleamide hydrolase also referred to as a fatty acid amide hydrolase or FAAH (SEQ ID NO 36). The encoded rat FAAH is appropriately abbreviated rFAAH. Bold type indicates the putative transmembrane spanning domain as predicted by PSORT. The seven discontinuous underlined regions indicate the seven separate peptides, the designation of which is consecutive, obtained by HPLC purification of a trypsin digest of the enzyme. The double-underlined segment is the putative SH3-domain-binding sequence.

Figures 10-1 through 10-5 show the continuous double-stranded cDNA sequence for rat FAAH as described in Section D. The encoded amino acid sequence is also indicated beginning with the ATG start site encoding methionine (M). The stop codon is also shown as boxed. The top and bottom strands of the cDNA sequence are respectively listed in SEQ ID NOs 35 and 37 with the amino acid sequence listed with the nucleotide sequence in SEQ ID NO 35 and by itself in SEQ ID NO 36.

Figure 11 illustrates the alignment of the amidase signature sequence region of the rat FAAH (SEQ ID NO 36 from amino acid residue 215 to and including 246) with several other representative amidases as further described in Section D1. Residues of the signature sequence that are completely conserved among the family members are shown in bold type and the relative amino acid position of the signature sequence of each member is given by the numbers just preceding and following the sequence information. From top to bottom, the sequences have the following respective SEQ ID Nos: 36 (from residue 215 to 246); 47, 48, 49, 50, 51, 52 and 53.

Figure 12A and 12B show the respective results of Southern and Northern blots as probed with an internal 800 bp fragment of rat FAAH cDNA as further described in Section D.

Figures 13-1 through 13-4 show the continuous double-stranded cDNA sequence for mouse FAAH as described in Section D2. The encoded amino acid sequence is also indicated beginning with the ATG start site encoding methionine (M). The stop codon is also shown as boxed. The top and bottom strands of the cDNA sequence are respectively listed in SEQ ID NOs 39 and 41 with the amino acid sequence listed with the nucleotide sequence in SEQ ID NO 39 and by itself in SEQ ID NO 40.

Figures 14-1 through 14-5 show the continuous double-stranded cDNA sequence for human FAAH as described in Section D3. The encoded amino acid sequence is also indicated beginning with the ATG start site encoding methionine (M). The stop codon is also shown as boxed. The top and bottom strands of the cDNA sequence are respectively listed in SEQ ID NOs 42 and 44 with the amino acid sequence listed with the nucleotide sequence in SEQ ID NO 42 and by itself in SEQ ID NO 43.

Figure 15A shows the expression of recombinant rat FAAH in COS-7 cells produced as described in Section E as performed by thin layer chromatography demonstrating the conversion of labeled oleamide to oleic acid as further described in Section F.

Figure 15B shows the inhibition of recombinant rat FAAH by trifluoromethyl ketone also performed as described in Figure 15A as further described in Section F.

Figure 15C shows the results of Western blotting of recombinant rat FAAH with antibodies generated against peptide 2 as shown in Figure 9 as shown in the four left lanes (1-4) and as competed with peptide 2 as shown in the four right lanes (5-8). Samples of untransfected COS-7 cell extract are shown in lanes 4 and 8, FAAH-transfected COS-7 cell extracts are shown in lanes 3 and 7, affinity-purified rat FAAH is shown in lanes 2

and 6 and a mixture of FAAH-transfected COS-7 cell extracts and affinity-purified FAAH is run in lanes 1 and 5. The proteins were probed with antibodies in the absence (lanes 1-4) or presence (lanes 5-8) of competing peptide antigen. The FAAH-transfected COS-7 cell extract but not the control contained an immunoreactive 60K-65K protein that was effectively competed away by preincubation of the antibodies with excess peptide antigen while the trace quantities of cross reactive protein observed in both transfected and untransfected COS-7 cell extracts were not competed by the peptide.

Figure 16 shows the ability of human recombinant expressed FAAH to hydrolyze oleamide to oleic acid, as further described in Figure 15A with thin layer chromatography and in Section F.

Figure 17 shows the results of thin layer chromatography demonstrating the conversion of labeled anandamide to arachidonic acid in rat FAAH-transfected COS-7 cells as shown in lane 3 but not in control untransfected cells (lane 2). TLC standards of anandamide and arachidonic acid are shown in lanes 1 and 4, respectively.

Detailed Description of the Invention

A. Protocols for the Induction of Sleep

Synthetic *cis*-9,10-octadecenoamide was injected (ip) into rats in order to test its effect on spontaneous behavior at different doses: 1 (n=2), 2 (n=2), 5 (n=7), 10 (n=10), 20 (n=2), and 50 (n=2) mg, where n = number of rats tested. Rats were injected during a reversed dark period (12:12) two hours after the lights cycled off and were observed in their home cages. With the lower doses (1 and 2 mg), no overt effect on spontaneous behavior was witnessed. However, at a threshold of

5 mg and above there was a marked effect consisting of an induction of long-lasting motor quiescence associated with eyes closed, sedated behavior characteristic of normal sleep. Also as with normal sleep, the rats still responded to auditory stimuli with orienting reflex and sustained attention toward the source of stimulation. In addition, motor behavior was impaired. The latency to behavioral sedation following administration was about 4 minutes and subjects were normally active again after 1 hour (5 mg), 2 hour (10 mg), or 2.5 hour (20 mg and 50 mg).

We have compared *cis*-9,10-octadecenoamide to vehicle and the synthetic analogs listed in Figure 1 to estimate the structural specificity of its sleep-inducing potential. Neither vehicle (5% ethanol in saline solution) nor oleic acid (**5**) showed any overt behavioral effect. *Trans*-9,10-octadecenoamide demonstrated similar pharmacological effects to its cis counterpart, but was much less potent as measured by the comparatively shorter duration time for its sleep-inducing properties (at 10 mg per rat, the biological effect lasted one hour for the trans isomer and two hours for the cis isomer). When the olefin was moved either direction along the alkyl chain (to the 8,9 (**3**) or 11,12 (**4**) positions) or the alkyl chain length was extended to 22 carbons (**6**), a substantial reduction in both the degree and duration of the pharmacological effects was observed, and while the mobility of the rats still decreased, their eyes remained open and their alertness appeared only slightly affected. Finally, polysomnographic studies on rats injected with *cis*-9,10-octadecenoamide show an increase in the total time of slow wave sleep (SWS) as well as in the mean duration of the SWS individual periods when compared to vehicle controls. More particularly, male Sprague-Dawley rats (300 g at

the time of surgery) were implanted under halothane anesthesia (2-3%) with a standard set of electrodes for sleep recordings. This included two screw electrodes placed in the parietal bone over the hippocampus to record the subjects electroencephalogram (EEG) and two wire electrodes inserted in the neck musculature to record postural tone through electromyographic activity (EMG). Rats were housed individually with at libitum access to food and water. The dark-light cycle was controlled (12:12, lights on a 10:00 p.m.). One week after the surgery, rats were habituated to the recording conditions for at least three days. Upon the completion of the habituation period, rats received 2 milliliter (ip) of either: vehicle (5% ethanol/saline solution), *cis*-9,10-octadecenoamide (10 mg), or oleic acid (10 mg). Rats were continuously recorded for four hours after the ip injection (12:00 p.m.-4:00 p.m.) Rats were observed for spontaneous changes in behavior through a one-way window. Sleep recordings were visually scored and four stages were determined: wakefulness, slow-wave-sleep 1 (SWS1), slow-wave-sleep 2 (SWS2), and rapid eye movement (REM) sleep.

These increases with respect to slow wave sleep (SWS) were at the expense of waking. Distribution of REM sleep does not seem to be altered. Together, these data suggest that *cis*-9,10-octadecenoamide could play an important role in slow-wave sleep modulation.

The traditional view of lipid molecules as passive structural elements of cellular architecture is rapidly giving way to an ever increasing awareness of the active roles these agents play in transducing cell signals and modifying cell behavior, e.g., Liscovitch et al, Cell, 77:329 (1994). An intriguing feature of the fatty acid amides studied here is that they belong to a family of simple molecules in which a great

deal of diversity may be generated by simply varying the length of the alkane chain and the position, stereochemistry, and number of its olefin(s). Interestingly, other neuroactive signalling molecules with amide modifications at their carboxy termini have been reported, from carboxamide terminal peptides to arachidonylethanolamide. Neuroactive signalling molecules employing carboxamide terminal peptides are disclosed by Eipper et al, Annu. Rev. Neurosci., 15:57 (1992). Neuroactive signalling molecules employing arachidonylethanolamide is disclosed by Devane et al, Science, 258:1946 (1992). It is disclosed herein that *cis*-9,10-octadecenoamide is a member of a new class of biological effectors in which simple variations of a core chemical structure have unique physiological consequences.

B. Isolation and assay of integral membrane protein fraction with FAAH activity

1. Observations on Lipid Amidase Activity

Lipid amidase activity has been observed in brain, liver, lung, kidney and spleen tissues, but not in heart tissue. The activity is inhibited by 1 mM PMSF (phenylmethylsulfonyl fluoride) and 50 mM HgCl, which is a test for sulfhydryl group dependency of the reaction. Since the fractions are not solubilized by 100 mM sodium carbonate (pH 11.5), the sample is apparently a membrane protein, which has been identified in nuclear, microsomal, and plasma membrane subcellular fractions, but not in the cytosol.

The enzyme catalyzed hydrolysis of *cis*-9,10-octadecenoamide to oleic acid by purified *cis*-9,10-octadecenoamide and inhibition of this enzyme by PMSF is disclosed on an

autoradiogram of a thin layer chromatographic plate (SiO_2, 55% ethyl acetate/hexanes), illustrated in Figure 6. In each case the enzymic reaction is performed is a separate reaction vessel and the product is spotted onto a TLC plate. The various reaction conditions for the reaction vessel corresponding to each lane are identified as follows:

lane 1: *Cis*-9,10-octadecenoamide standard;

lane 2: *Cis*-9,10-octadecenoamide with rat brain soluble fraction;

lane 3: *Cis*-9,10-octadecenoamide with rat brain membrane fraction;

lane 4: *Cis*-9,10-octadecenoamide with rat brain membrane fraction + 1 mM PMSF;

lane 5: *Cis*-9,10-octadecenoamide with rat brain membrane fraction + 5 mM EDTA;

lane 6: *Cis*-9,10-octadecenoamide with rat pancreatic microsomes;

lane 7: *Cis*-9,10-octadecenoamide with proteinase K (200 mg); and

lane 8: oleic acid standard.

Inhibition studies of *Cis*-9,10-octadecenoamide hydrolysis to oleic acid with $HgCl_2$ are illustrated in Figure 7. Between 50 mM and 5 mM $HgCl_2$ lies the optimal concentrations required for inhibition of amide hydrolysis activity. The enzyme catalyzed hydrolysis of *cis*-9,10-octadecenoamide to oleic acid by purified *cis*-9,10-octadecenoamide and inhibition of this enzyme by $HgCl_2$ is performed in a series of reaction vessels and spotted onto a thin layer chromatographic plate (SiO_2, 55% ethyl acetate/hexanes). A typical $HgCl_2$ inhibition study uses a 100 mM

$HgCl_2$ stock (27 mg in 1mL Tris buffer (50 mM), pH 7.5) of $HgCl_2$. The various reaction conditions for the reaction vessels corresponding to each lane are identified as follows:

lane 1: *Cis*-9,10-octadecenoamide standard;

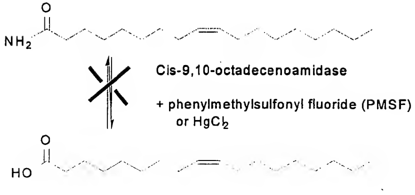
lane 2: *Cis*-9,10-octadecenoamide with rat brain soluble fraction;

lane 3: *Cis*-9,10-octadecenoamide with rat brain membrane fraction and 500 mM $HgCl_2$;

lane 4: *Cis*-9,10-octadecenoamide with rat brain membrane fraction and 50 mM $HgCl_2$;

lane 5: *Cis*-9,10-octadecenoamide with rat brain membrane fraction and 5 mM $HgCl_2$;

lane 6: oleic acid standard.



SCHEME 2

A unique enzymatic activity capable of degrading the putative effector molecule, *cis*-9,10- octadecenoamide has been identified and is disclosed herein. Rapid conversion of ^{14}C-cis-9,10- octadecenoamide to oleic acid by rat brain membrane fractions was observed by TLC. The enzymatic activity was unaffected by 5 mM EDTA, but was completely inhibited by 1 mM phenylmethylsulfonyl fluoride (PMSF). Only trace amide hydrolysis activity was observed with rat brain soluble fractions, while rat pancreatic microsomes and proteinase K showed no significant capacity to hydrolyze *cis*-9,10-octadecenoamide to oleic acid.

2. <u>Synthesis of fatty acid primary amides</u>

Preferred protocols for synthesizing exemplary

fatty acid primary amides are provided. The synthetic protocols differ only with respect to the chain length of the starting materials, the product yields, and the separation of the various *cis* and *trans* products. Accordingly, exemplary descriptions of synthetic protocols for the synthesis of *cis*-9,10-octadecenoamide and several other fatty acid primary amides are provided and serve to illustrate the synthetic protocol for the entire class of fatty acid primary amides.

3. Isolation of rat integral membrane protein fraction with FAAH activity

The protocol described herein is for about 5-10 g of tissue. The rat liver(s) are collected, weighed and then placed in 1mM $NaHCO_3$ on ice. Next, the liver is cut up, rinsed (2X) with 1mM $NaHCO_3$ and minced with a razor blade on a sheet of wax. It is then placed into 25 ml of 1mM sodium bicarbonate and homogenized in a tissuemizer for 2 minutes at setting 6. A dilution to 100 ml with 1mM sodium bicarbonate is subsequently performed, which is followed by a filtration through 4 layers of cheesecloth and then through 8 layers. The filtrate is then brought up to 100 ml and split into four JA-20 tubes and topped off with 1 mM sodium bicarbonate. The tubes are spun at 6,000 rpm (4500 x g) for 12 minutes at 4°C in the JA-20 rotor. Using a Pasteur pipette, the fat layer is sucked off and the supernatant layer is decanted and saved.

Next, the pellet is resuspended in the remaining supernatant layer with a syringe and needle. 20 mL fractions of the resuspension are then dounced 16 times with a 15 ml dounce homogenizer. The fractions are then combined into a single JA-20 tube and brought up to volume with 1 mM $NaHCO_3$. The tubes are next spun again at 6,000 rpm (4500 x g) for 15 minutes at 4°C in

a JA-20 rotor and the supernatant is subsequently poured off and saved. The pellet is resuspended and dounced as before and then brought up to 10 ml volume with 1mM sodium bicarbonate. Next, 20 mL of 67% sucrose solution is added to a final volume of 30 ml and the mixture is split into 2 tubes. An additional 25 mL of 30% sucrose is added to the top of each tube and spun at 27 K rpm for 1 hour 45 minutes at 4°C in an ultracentrifuge. The fractions are collected from the sucrose gradient and the middle band from the sucrose gradient (plasma membrane band) is placed in a capped plastic tube and filled with 1 mM sodium bicarbonate. The tube is subsequently spun at 17,000 rpm for 35 minutes at 4°C.

The supernatant is discarded and the pellets are resuspended (with Douncing) in 100 mM of sodium carbonate. This solution is subsequently kept on ice for 1 hour and then spun at 100,000 g for 1 hour. The supernatant (solubilized peripheral membrane proteins) is discarded since no lipid amidase activity is present in this fraction and the pellet is resuspended (with Douncing) in 10% glycerol, 1% Triton, 0.1% phosphatidyl choline, 20 mM Hepes buffer and then stirred for two hours at 4°C. Finally the solution is spun at 100,000 g for 1 hour and the supernatant thus obtained is further purified as follows.

4. <u>Purification via 4 step column chromatography process</u>

Step 1 DEAE column/ ion exchange (Figure 3). The above solubilized supernatant batch is further purified. The supernatant batch is mixed with DEAE-Sephadex (Diethylaminoethyl-Sephadex, commercially available from Sigma chemical company) ion exchange resin for 1 hour at 4°C. The fraction which is bound to the DEAE resin, displays the lipid

amidase activity (none in flow through). Solubilized rat liver plasma membrane (in BI: 10% glycerol, 1% Triton X-100, 1 mM EDTA, 20 mM Hepes, pH 7.2) is passed over DEAE Fast Flow column (Pharmacia) and washed with 5 column volumes of BI, 0.2% Triton. Then the amidase activity is eluted with 1 column volume each of 50 mM, 100 mM, and 200 mM NaCl in BI with 0.2% Triton.

Step 2 Hg Column (Figure 3). The above eluent from the DEAE exchange, is mixed with p-chloromercuric benzoic acid resin (Commercially available from BioRad chemical company) for 1 hour at 4°C. The fraction which is bound to the above mercury resin, displays the lipid amidase activity (none in flow through), is washed with 5 column volumes of BI with 0.2% Triton, 5 column volumes of BI with 0.2% Triton and 150 mM NaCl, and eluted with 1.5 column volumes BI with 0.2% Triton, 150 mM NaCl, and 25 mM b-mercaptoethanol.

Step 3 Heparin column (Figure 3). Hg-eluted amidase activity was passed over Heparin column (BioRad) and washed with 10 column volumes of BI with 0.7% CHAPS and 150 mM NaCl (detergent exchange). Elution was conducted with 1 column volume each of BI with 0.7% CHAPS and 300 mM, 400 mM, 500 mM, 650 mM, and 750 mM NaCl,respectively, with amidase activity eluting in the final two fractions.

Step 4 Affinity column (Figures 3 and 4). Heparin-eluted amidase activity was mixed with Triton X-100 for a final concentration of 0.2%, and then passed over CF_3-inhibitor linked to activated pyridyl disulphide beads (**103**: attachment of inhibitor to beads is described infra) and washed with 20 column volumes of BI with 0.2% Triton X-100. Elution was conducted by passing 3 column volumes of BI with 0.2% Triton and 20 mM DTT, and letting column stand at 4o C for 30 h. Then, washing column with 1.5 column volumes of BI with 0.2% Triton and 20 mM DTT

eluted single protein of 60 kD in size.

Eluted 60 kd protein was digested with trypsin and peptides were sequenced as described infra.

The purity of the activity is then assessed after this procedure according to an assay protocol.

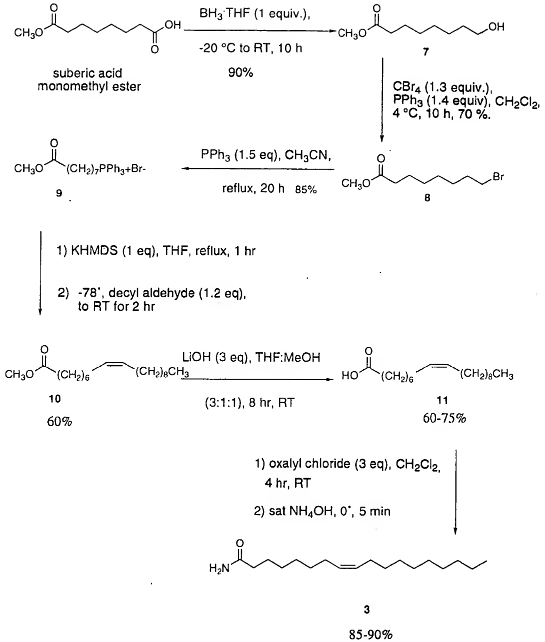
5. Assay for Fatty-Acid Amide Hydrolase Activity:

The following thin layer chromatography (TLC) protocol is used for assaying *cis*-9,10 octadecenoamide hydrolysis activity, also referred to as fatty-acid amide hydrolase activity. Oleamide is first labeled with ^{14}C. To accomplish this, ^{14}C-Oleic acid (1-10 μM, Moravek Biochemicals, 5-50 μCi/μM) in CH_2CL_2 (200 μL, 0.005-0.05 M) at 0°C was treated with excess oxalyl chloride and the reaction mixture was warmed to 25°C for 6 hours. The reaction mixture was then concentrated under a constant stream of gaseous nitrogen and the remaining residue was cooled to 0°C and treated with excess saturated aqueous ammonium hydroxide. After 5 minutes, the reaction mixture was partitioned between Et)Ac (1.5 mL) and 10% HCl (1.0 mL). The organic layer was then washed with water (1.0 mL) and concentrated under a constant stream of gaseous nitrogen to provide ^{14}C-oleamide in quantitative yield as judged by TLC (60% EtOAc in hexanes; oleamide R_f-0.2; oleic acid R_f-0.8).

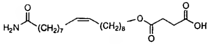
Approximately 1 μCi of ^{14}C-oleamide (specific activity 5-50 μCi/μM) in ethanol was incubated at 37°C for 1-2 hours with 70 μL of 126 mM Tris-HCl, pH 9.0 (final concentration of ethanol was 2.0%). The reaction mixture was then partitioned between ethyl acetate (1.0 mL) and 0.07 M Hcl (0.6 mL). The ethyl acetate layer was concentrated under a constant stream of gaseous nitrogen and the remaining residue was resuspended in 15 μL of ethanol. Approximately 3 μL of this ethanol stock was

then used for TLC analysis (60% EtOAc in hexanes: oleamide R_f-0.2; oleic acid R_f-0.8). Following exposure to solvent, TLC plates were air-dired, treated with EN^{3}HANCE spray (Dupont NEN) according to manufacturer's guidelines and exposed to film at -78°C for 1-2 hours.

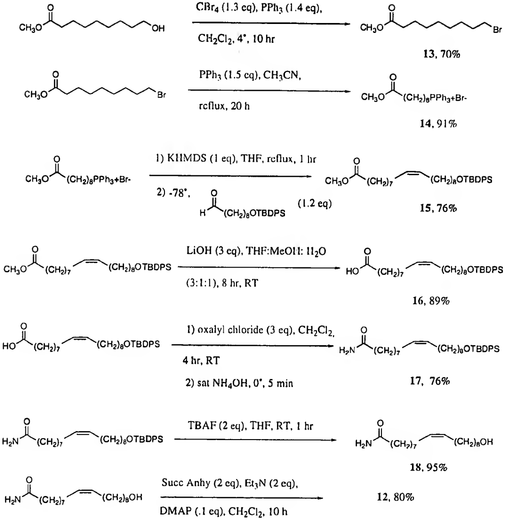
The purified protein was determined to have a 20-30 fold enrichment of amidase activity from crude membrane protein fraction by visual comparison of the purified protein band intensity with the crude protein fraction. Estimated purified yield is 10-15% (Figure 3).


BH3·THF (1 equiv.),
-20 °C to RT, 10 h
90%
suberic acid monomethyl ester
7
CBr4 (1.3 equiv.), PPh3 (1.4 equiv), CH2Cl2, 4 °C, 10 h, 70 %.
8
PPh3 (1.5 eq), CH3CN,
reflux, 20 h 85%
9
1) KHMDS (1 eq), THF, reflux, 1 hr
2) -78°, decyl aldehyde (1.2 eq), to RT for 2 hr
10
60%
LiOH (3 eq), THF:MeOH
(3:1:1), 8 hr, RT
11
60-75%
1) oxalyl chloride (3 eq), CH2Cl2, 4 hr, RT
2) sat NH4OH, 0°, 5 min
3
85-90%

Scheme 3



12


CBr4 (1.3 eq), PPh3 (1.4 eq),
CH2Cl2, 4°, 10 hr
13, 70%
PPh3 (1.5 eq), CH3CN,
reflux, 20 h
14, 91%
1) KHMDS (1 eq), THF, reflux, 1 hr
2) -78°,
(1.2 eq)
15, 76%
LiOH (3 eq), THF:MeOH: H2O
(3:1:1), 8 hr, RT
16, 89%
1) oxalyl chloride (3 eq), CH2Cl2,
4 hr, RT
2) sat NH4OH, 0°, 5 min
17, 76%
TBAF (2 eq), THF, RT, 1 hr
18, 95%
Succ Anhy (2 eq), Et3N (2 eq),
DMAP (.1 eq), CH2Cl2, 10 h
12, 80%

Scheme 4

C. Synthetic Protocols

1. Cis-9,10-octadecenoamide (1; Figure 1):

A solution of oleic acid (1.0 g, 3.55 mmol, 1.0 equiv.) in CH_2Cl_2 (8.9 mL, 0.4 M) at 0 °C was treated dropwise with oxalyl chloride (5.32 mL, 2.0 M solution in CH_2Cl_2, 10.64 mmol, 3.0 equiv.). The reaction mixture was stirred at 25 °C for 4 h, concentrated under reduced pressure, cooled to 0 °C, and treated with saturated aqueous NH_4OH (2.0 mL). The reaction mixture was then partitioned between ethyl acetate (EtOAc) (100 mL) and H_2O (100 mL), and the organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. Chromatography (SiO_2, 5 cm x 15 cm, 40-100% EtOAc-hexanes gradient elution) afforded 1 as a white solid (0.810 g, 0.996 g theoretical, 81.3%): ^1H NMR ($CDCl_3$, 250 MHz) δ 6.06 (bs, 1H, $NH_2C(O)$), 5.58 (bs, 1H, $NH_2C(O)$), 5.32 (m, 2H, $CH{=}CH$), 2.16 (t, 2H, J = 7.5 Hz, $CH_2C(O)NH_2$), 2.02 (m, 4H, $CH_2CH{=}CHCH_2$), 1.61 (m, 2H, $CH_2CH_2C(O)NH_2$), 1.29 (b s, 14H, alkyl protons), 0.87 (t, 3H, CH_3); FABHRMS (NBA/NaI *m/e* 282.2804 ($C_{18}H_{35}NO + H^+$ requires 282.2797). The regions of the spectra that distinguish between the *cis* and *trans* isomers are the olefinic protons from δ 5.3 to 5.2 and allylic protons from δ 2.0 to 1.8. These regions identify the natural compound as cis-9,10-octadecenoamide.

2. Trans-9,10-octadecenoamide (2; Figure 1)

A solution of elaidic acid (1.0 g, 3.55 mmol, 1.0 equiv.) in CH_2Cl_2 (8.9 mL, 0.4 M) at 0 °C was treated dropwise with oxalyl chloride (5.32 mL, 2.0 M solution in CH_2Cl_2, 10.64 mmol, 3.0 equiv.). The reaction mixture was stirred at 25 °C for 4 h, concentrated under reduced pressure, cooled to 0 °C, and treated with saturated aqueous NH_4OH (2.0 mL). The reaction

mixture was then partitioned between ethyl acetate (EtOAc) (100 mL) and H_2O (100 mL), and the organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. Chromatography (SiO_2, 5 cm x 15 cm, 40-100% EtOAc-hexanes gradient elution) afforded 2 as a white solid. The regions of the spectra that distinguish between the *cis* and *trans* isomers are the olefinic protons from δ 5.3 to 5.2 and allylic protons from δ 2.0 to 1.8. These regions identify the compound as trans-9,10-octadecenoamide.

3. Cis-8,9-octadecenoamide (3: Figure 1):

A solution of 11, synthesized infra, (0.130 g, 0.461 mmol, 1.0 equiv.) in CH_2Cl_2 (1.5 mL, 0.31 M) at 0 °C was treated dropwise with oxalyl chloride (0.69 mL, 2.0 M solution in CH_2Cl_2, 1.38 mmol, 3.0 equiv.). The reaction mixture was stirred at 25 °C for 4 h, concentrated under reduced pressure, cooled to 0 °C, and treated with saturated aqueous NH_4OH (2.0 mL). The reaction mixture was then partitioned between ethyl acetate (EtOAc) (100 mL) and H_2O (100 mL), and the organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. Chromatography (SiO_2, 5 cm x 15 cm, 40-100% EtOAc-hexanes gradient elution) afforded 3 as a white solid. (0.105 g, 0.130 theoretical, 80.8%): ^1H NMR ($CDCl_3$, 250 MHz) δ 5.70-5.34 (m, 4H, $H_2NC(O)$ and CH=CH), 2.21 (t, 2H, J = 7.5 Hz, $CH_2C(O)NH_2$), 2.00 (m, 4H, $CH_2CH=CHCH_2$), 1.63 (m, 2H, $CH_2CH_2C(O)NH_2$), 1.47-1.23 (m, 20H, alkyl protons), 0.87 (t, 3H, RCH_3); FABHRMS (NBA/CSI *m/e* 414.1762 ($C_{18}H_{35}NO + Cs^+$ requires 414.1773).

4. Cis-11,12-octadecenoamide (4: Figure 1):

A solution of Δ11,12 octadecenoic acid (1.0 g, 3.55 mmol, 1.0 equiv.) in CH_2Cl_2 (8.9 mL, 0.4 M) at 0 °C was treated dropwise with oxalyl chloride (5.32 mL, 2.0 M solution

in CH_2Cl_2, 10.64 mmol, 3.0 equiv.). The reaction mixture was stirred at 25 °C for 4 h, concentrated under reduced pressure, cooled to 0 °C, and treated with saturated aqueous NH_4OH (2.0 mL). The reaction mixture was then partitioned between ethyl acetate (EtOAc) (100 mL) and H_2O (100 mL), and the organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. Chromatography (SiO_2, 5 cm x 15 cm, 40-100% EtOAc-hexanes gradient elution) afforded 4 as a white solid.

5. Oleic acid (5: Figure 1)

Oleic acid was obtained from Aldrich chemical company, CAS #112-80-1.

6. Erucamide (6: Figure 1)

Erucamide was obtained from Aldrich Chemical Company, CAS #28,057-7.

7. Methyl-8-hydroxy-octanoate (7: Scheme 3)

A solution of suberic acid monomethyl ester (1.5 g, 7.97 mmol, 1.0 equiv.) in tetrahydrofuran (THF) (32.0 mL, .25M) at -20 °C was treated dropwise with BH_3.THF (1M solution in THF, 7.97 mL, 7.97 mmol, 1.0 equiv.). The reaction mixture was stirred overnight and was subsequently allowed to reach room temperature. The reaction mixture was then diluted with ethyl acetate (100 mL) and quenched with methanol (10 mL) and 10% HCl (10 mL). Extraction with $NaHCO_3$ (1X 20 mL), water (2X 10 mL), and brine (1X 10 mL), afforded methyl-8-hydroxy-octanoate (7) as a crude white solid.

8. Methyl-8-bromo-octanoate (8: Scheme 3)

A solution of crude methyl-8-hydroxy-octanoate

(7, 1.24 g, 7.13 mmol, 1.0 equiv.) in CH_2Cl_2 (15 mL, 0.48 M) at 0 °C was treated successively with CBr_4 (3.07 g, 9.27 mmol, 1.3 equiv.) and PPh_3 (2.61 g, 9.98 mmol, 1.4 equiv.) and the reaction mixture was stirred at 4 °C for 10 h. The reaction mixture was then concentrated under reduced pressure and washed repeatedly with Et_2O (8 x 10 mL washes). The Et_2O washes were combined and concentrated under reduced pressure. Chromatography (SiO_2, 5 cm x 15 cm, hexanes) afforded 8 as a clear, colorless oil (1.25 g, 1.69 g theoretical, 74.0%): ^1H NMR ($CDCl_3$, 250 MHz) δ 3.64 (s, 3H, $C(O)OCH_3$), 3.38 (t, 2H, J = 6.8 Hz, CH_2Br), 2.29 (t, 2H, J=7.4 Hz $CH_2C(O)OCH_3$), 1.83 (p, 2H, CH_2CH_2Br), 1.63 (m, 2H, $CH_2CH_2C(O)OCH_3$) 1.47-1.28 (m, 6H, alkyl protons).

9. <u>Methyl-8-triphenylphosphoranyl-octanoate-bromide (9: Scheme 3)</u>

A solution of 8 (1.25 g, 5.23 mmol, 1.0 equiv.) in CH_3CN (4.0 mL, 1.31 M) was treated with triphenylphosphine (1.52 g, 5.75 mmol, 1.1 equiv.) and stirred at reflux for 10 h. Additional triphenylphosphine (0.685 g, 2.61 mmol, 0.5 equiv.) was added to the reaction mixture and stirring was continued at reflux for 5 h. The reaction mixture was concentrated under reduced pressure and washed repeatedly with Et_2O (5 x 10 mL washes). The remaining residue was then solubilized in the minimum volume of CH_2Cl_2 and concentrated under reduced pressure to afford 9 as a colorless foam (2.20 g, 2.61 g theoretical, 84.3%): 1H NMR ($CDCl_3$, 250 MHz) δ 7.82-7.51 (m, 15H, ArH), 3.70-3.46 (m, 5H, $CH_3OC(O)R$ and CH_2PPh_3), 2.13 (t, 2H, J = 7.4 Hz, $CH_2C(O)OCH_3$), 1.62-1.43 (m, 6H, alkyl protons), 1.30-1.02 (m, 4H, alkyl protons); FABHRMS (NBA) *m/e* 419.2154 ($C_{27}H_{32}BrO_2P-Br^-$ requires 419.2140).

10. Methyl-*cis*-8,9-octadecenoate (10: Scheme 3)

A solution of 9 (0.71 g, 1.42 mmol, 1.0 equiv.) in THF (7.0 mL, 0.2 M) at 25 °C was treated with KHMDS (3.0 mL, 0.5 M solution in THF, 1.5 mmol, 1.06 equiv.) and the reaction mixture was stirred at reflux for 1 h. The reaction mixture was then cooled to -78 °C, treated with decyl aldehyde (0.321 mL, 1.71 mmol, 1.2 equiv.) warmed to 25 °C, and stirred for an additional 30 min. The reaction mixture was then treated with saturated aqueous NH_4Cl and partitioned between EtOAc (100 mL) and H_2O (100 mL). The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. Chromatography (SiO_2, 5 cm x 15 cm, 0-2% EtOAc-hexanes gradient elution) afforded 10 as a colorless oil (0.290 g, 0.422 g theoretical, 68.7 %): ^1H NMR ($CDCl_3$, 250 MHz) δ 5.34 (m, 2H, CH=CH), 3.65 (s, 3H, $CH_3OC(O)$), 2.29 (t, 2H, J = 7.4 Hz, $CH_2C(O)OCH_3$), 2.00 (m, 4H, $CH_2CH{=}CHCH_2$), 1.61 (m, 2H, $CH_2CH_2C(O)OCH_3$), 1.29 (bs, 20 H, alkyl protons), 0.86 (t, 3H, RCH_3).

11. Cis-8,9 octadecenoic acid (11: Scheme 3)

A solution of 10 (0.245 g, 0.825 mmol, 1.0 equiv.) in THF-MeOH-H_2O (3-1-1 ratio, 4.1 mL, 0.2 M) at 0 °C was treated with $LiOH{\cdot}H_2O$ (0.104 g, 2.48 mmol, 3.0 equiv.). The reaction mixture was warmed to 25 °C, stirred for 8 h, and then partitioned between EtOAc (100 mL) and H_2O (100 mL). The organic layer was washed successively with 10% aqueous HCl (100 mL) and saturated aqueous NaCl (100 mL), dried, and concentrated under reduced pressure. Chromatography (SiO_2, 5cm x 15 cm, 10-30% EtOAc-hexanes gradient elution) afforded 11 as a colorless oil (0.156 g, 0.233 g theoretical, 67.0%): ^1H NMR ($CDCl_3$, 250 MHz) δ 5.34 (m, 2H, CH=CH), 2.34 (t, 2H, J = 7.4 Hz, CH_2COOH), 2.01 (m, 4H, $CH_2CH{=}CHCH_2$), 1.61 (m, 2H, CH_2CH_2COOH), 1.47-1.23 (m, 20 H,

alkyl protons), 0.87 (t, 3H, RCH_3).

12. <u>18-Hemisuccinate-*cis*-9,10-octadecenoamide (12; Scheme 4)</u>

A solution of 18 (0.047 g, 0.160 M, 1.0 equiv) in CH_2Cl_2-$CHCl_3$ (3-1, 1.60 mL, 0.1M) was treated successively with Et_3N (0.045 mL, 0.320 mmol, 2.0 equiv), succinic anhydride (0.033 g, 0.320 mmol, 2.0 equiv) and DMAP (0.002 g, 0.016 mmol, 0.1 equiv), and the reaction mixture was stirred at 25 °C for 10 h. The reaction mixture was then partitioned between CH_2Cl_2 (50 mL) and H_2O (50 mL), and the organic layer was washed successively with 10% aqueous HCl (50 mL) and saturated aqueous NaCl (50 mL), dried (Na_2SO_4), and concentrated under reduced pressure. Chromatography (SiO_2, 3 cm x 15 cm, 0-10% MeOH-EtOAc) afforded 12 as a white solid (0.051 g, 0.063 theoretical, 80.3%): ^{1}H NMR ($CDCl_3$, 250 MHz) δ 6.95 (b s, 1H, $H_2NC(O)$), 5.72 (b s, 1H, $H_2NC(O)$), 5.34 (m, 2H, CH=CH), 4.08 (t, 3H, J = 6.6 Hz, $CH_2OC(O)R$), 2.61 (m, 4H, $ROC(O)CH_2CH_2COOH$), 2.21 (t, 2H, J = 7.5 Hz, $CH_2C(O)NH_2$), 2.00 (m, 4H, CH_2CH=$CHCH_2$), 1.70-1.52 (m, 4H, $CH_2CH_2C(O)NH_2$ and CH_2CH_2OH), 1.29 (b s, 18H, alkyl protons); FABHRMS (NBA) m/e 398.2893 ($C_{22}H_{39}NO_5$ + H^+ requires 398.2906).

13. <u>Methyl-9-bromo-nonanoate (13; Scheme 4)</u>

A solution of methyl-9-hydroxy-nonanoate (1.1 g, 5.85 mmol, 1.0 equiv) in CH_2Cl_2 (30 mL, 0.2 M) at 0 °C was treated successively with CBr_4 (2.5 g, 7.54 mmol, 1.3 equiv) and PPh_3 (2.15 g, 8.19 mmol, 1.4 equiv) and the reaction mixture was stirred at 4 °C for 10 h. The reaction mixture was then concenctrated under reduced pressure and washed repeatedly with Et_2O (8 x 10 mL washes). The Et_2O washes were combined and concentrated under reduced pressure. Chromatography (SiO_2, 5 cm

x 15 cm, hexanes) afforded 13 as a clear, colorless oil (1.02 g, 1.47 g theorectical, 69.5 %): ^{1}H NMR ($CDCl_3$, 250 MHz) d 3.64 (s, 3H, C(O)OCH_3), 3.38 (t, 2H, J = 6.8 Hz, CH_2Br), 2.29 (t, 2H, J = 7.4 Hz CH_2C(O)OCH_3), 1.83 (p, 2H, CH_2CH_2Br), 1.63 (m, 2H, CH_2CH_2C(O)OCH_3) 1.47-1.28 (m, 8H, alkyl protons).

14. Methyl-9-triphenylphosphoranyl-nonanoate-bromide (14: Scheme 4)

A solution of 13 (1.02 g, 4.06 mmol, 1.0 equiv) in CH_3CN (3.5 mL, 1.16 M) was treated with triphenylphosphine (1.17 g, 4.47 mmol, 1.1 equiv) and stirred at reflux for 10 h. Additional triphenylphosphine (0.532 g, 2.03 mmol, 0.5 equiv) was added to the reaction mixture and stirring was continued at reflux for 5 h. The reaction mixture was concentrated under reduced pressure and washed repeatedly with Et_2O (5 x 10 mL washes). The remaining residue was then solubilized in the minimum volume of CH_2Cl_2 and concentrated under reduced pressure to afford 14 as a colorless foam (1.90 g, 2.08 g theoretical, 91.3%): ^{1}H NMR ($CDCl_3$, 250 MHz) δ 7.82-7.51 (m, 15H, ArH), 3.70-3.46 (m, 5H, CH_3OC(O)R and CH_2PPh_3), 2.13 (t, 2H, J = 7.4 Hz, CH_2C(O)OCH_3), 1.62-1.02 (m, 12H, alkyl protons); FABHRMS (NBA) m/e 433.2312 ($C_{28}H_{34}BrO_2P$ - Br^- requires 433.2296).

15. Methyl-18-t-butyldiphenysilyloxy-*cis*-9,10 octadecenoate (15: Scheme 4)

A solution of 14 (1.0 g, 1.95 mmol, 1.0 equiv) in THF (6.5 mL, 0.3 M) at 25 °C was treated with KHMDS (3.9 mL, 0.5 M solution in THF, 1.95 mmol, 1.0 equiv) and the reaction mixture was stirred at reflux for 1 h. The reaction mixture was then cooled to -78 °C, treated with 3 (0.93 g, 2.35 mmol, 1.2 equiv), warmed to 25 °C, and stirred for an additional 30 min.

The reaction mixture was then treated with saturated aqueous NH_4Cl and partitioned between EtOAc (100 mL) and H_2O (100 mL). The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. Chromatography (SiO_2, 5 cm x 15 cm, 0-2% EtOAc-hexanes gradient elution) afforded 15 as a colorless oil (0.82 g, 1.07 g theoretical, 76.3%): ^{1}H NMR ($CDCl_3$, 250 MHz) δ 7.67 (m, 4H, ArH), 7.41 (m, 6H, ArH), 5.34 (m, 2H, C*H*=C*H*), 3.65 (m, 5H, C*H*$_3$OC(O) and C*H*$_2$OTBDPS), 2.29 (t, 2H, *J* = 7.4 Hz, C*H*$_2$C(O)OCH3), 2.00 (m, 4H, C*H*$_2$CH=CHC*H*$_2$), 1.55 (m, 4H, C*H*$_2$CH$_2$C(O)OCH$_3$ and C*H*$_2$CH$_2$OTBDPS), 1.29 (b s, 18H, alkyl protons), 1.04 (s, 9H, (C*H*$_3$)$_3$C).

16. <u>18-*T*-butyldiphenylsilyloxy-*cis*-9,10-octadecenoic acid (16: Scheme 4)</u>

A solution of 5 (0.81 g, 1.47 mmol, 1.0 equiv) in THF-MeOH-H_2O (3-1-1 ratio, 7.3 mL, 0.2 M) at 0 °C was treated with LiOH·H_2O (0.188 g, 4.48 mmol, 3.0 equiv). The reaction mixture was warmed to 25 °C, stirred for 8 h, and then partitioned between EtOAc (100 mL) and H_2O (100 mL). The organic layer was washed successively with 10% aqueous HCl (100 mL) and saturated aqueous NaCl (100 mL), dried, and concentrated under reduced pressure. Chromatography (SiO_2, 5 cm x 15 cm, 10-30% EtOAc-hexanes gradient elution) afforded 16 as a colorless oil (0.700 g, 0.790 g theoretical, 88.7%): ^{1}H NMR ($CDCl_3$, 250 MHz) δ 7.67 (m, 4H, ArH), 7.41 (m, 6H, ArH), 5.34 (m, 2H, C*H*=C*H*), 3.65 (t, 3H, J = 6.5 Hz, C*H*$_2$OTBDPS), 2.34 (t, 2H, *J* = 7.4 Hz, C*H*$_2$COOH), 2.00 (m, 4H, C*H*$_2$CH=CHC*H*$_2$), 1.65-1.50 (m, 4H, C*H*$_2$CH$_2$COOH and C*H*$_2$CH$_2$OTBDPS), 1.47-1.23 (m, 18H, alkyl protons), 1.05 (s, 9H, (C*H*$_3$)$_3$C); FABHRMS (NBA/CsI) *m/e* 669.2772 ($C_{34}H_{52}O_3Si$ + Cs^+ requires 669.2740).

17. 18-T-butyldiphenylsilyloxy-*cis*-9,10-octadecenoamide (17: Scheme 4)

A solution of 16 (0.685 g, 1.28 mmol, 1.0 equiv) in CH_2Cl_2 (4.3 mL, 0.3 M) at 0 °C was treated dropwise with oxalyl chloride (1.92 mL, 2 M solution in CH_2Cl_2, 3.84 mmol, 3.0 equiv). The reaction mixture was stirred at 25 °C for 4 h, concentrated under reduced pressure, cooled to 0 °C, and treated with saturated aqueous NH_4OH (2.0 mL). The reaction mixture was then partitioned between EtOAc (100 mL) and H_2O (100 mL), and the organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. Chromatography (SiO_2, 5 cm x 15 cm, 40-100% EtOAc-hexanes gradient elution) afforded 17 as a colorless oil (0.520 g, 0.684 g, 76.0%): ^1H NMR ($CDCl_3$, 250 MHz) δ 7.67 (m, 4H, ArH), 7.41 (m, 6H, ArH), 5.70-5.34 (m, 4H, $H_2NC(O)$ and $CH{=}CH$), 3.65 (t, 3H, J = 6.5 Hz, CH_2OTBDPS), 2.21 (t, 2H, J = 7.5 Hz, $CH_2C(O)NH_2$), 2.00 (m, 4H, $CH_2CH{=}CHCH_2$), 1.65-1.50 (m, 4H, $CH_2CH_2C(O)NH_2$ and CH_2CH_2OTBDPS), 1.47-1.23 (m, 18H, alkyl protons), 1.05 (s, 9H, $(CH_3)_3C$); FABHRMS (NBA/CsI *m/e* 668.2929 ($C_{34}H_{53}O_2NSi$ + Cs^+ requires 668.2900).

18. 18-Hydroxy-*cis*-9,10-octadecenoamide (18: Scheme 4)

A solution of 17 (0.185 g, 0.345 mmol, 1.0 equiv) in THF (1.1 mL, 0.31 M) was treated with tetrabutylammoniumfluoride (0.69 mL, 1.0 M solution in THF, 0.69 mmol, 2.0 equiv) and the reaction mixture was stirred at 25 °C for 2 h. The reaction mixture was then partitioned between EtOAc (50 mL) and H_2O (50 mL), and the organic layer was dried (Na_2SO_4) and concentrated under reduced pressure . Chromatography (SiO_2, 3 cm x 15 cm, 0-5% MeOH-EtOAc gradient elution) afforded 18 as a white solid (0.097 g, 0.103 g

theoretical, 94.6%): ^1H NMR ($CDCl_3$, 250 MHz) δ 5.65-5.34 (m, 4H, $H_2NC(O)$ and $CH{=}CH$), 3.62 (t, 3H, J = 6.5 Hz, CH_2OH), 2.21 (t, 2H, J = 7.5 Hz, $CH_2C(O)NH_2$), 2.00 (m, 4H, $CH_2CH{=}CHCH_2$), 1.65-1.50 (m, 4H, $CH_2CH_2C(O)NH_2$ and CH_2CH_2OH), 1.29 (b s, 18H, alkyl protons); FABHRMS (NBA) 298.2732 ($C_{18}H_{35}NO_2$ + H^+ requires 298.2746).

19. Synthesis of Compound 100 (Figure 5)

Methyl-9-*t*-butyldiphenylsilyloxy-nonanoate (intermediate for compound 100: Figure 5). A solution of methyl-9-hydroxy-nonanoate (0.838 g, 4.46 mmol, 1.0 equiv: Aldrich) in CH_2Cl_2 (15 mL, 0.3 M) was treated successively with Et_3N (0.75 mL, 5.38 mmol, 1.2 equiv), t-butylchlorodiphenylsilane (1.28 mL, 4.93 mmol, 1.1 equiv), and DMAP (0.180 g, 1.48 mmol, 0.33 equiv), and the reaction mixture was stirred at 25 °C for 12 h. Saturated aqueous NH_4Cl was added to the reaction mixture and the mixture was partitioned between CH_2Cl_2 (100 mL) and H_2O (100 mL). The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. Chromatography (SiO_2, 5 cm x 15 cm, 0-5% EtOAc-hexanes gradient elution) afforded the intermeidate as a clear, colorless oil (1.22g, 1.831 theoretical, 64.1%): ^1H NMR ($CDCl_3$, 250 MHz) δ 7.66 (m, 4H, ArH), 7.38 (m, 6H, ArH), 3.67-3.62 (m, 5H, $C(O)OCH_3$ and $CH_2OTBDPS$), 2.30 (t, 2H, J = 7.4 Hz, $CH_2C(O)OCH_3$), 1.58 (m, 4H, $CH_2CH_2OTBDPS$ and $CH_2CH_2C(O)OCH_3$), 1.28 (b s, 8H, alkyl protons), 1.05 (s, 9H, $C(CH_3)_3$)

20. Methyl-9-bromo-nonanoate (intermediate for compound 100: Figure 5)

A solution of methyl-9-hydroxy-nonanoate (1.1 g, 5.85 mmol, 1.0 equiv) in CH_2Cl_2 (30 mL, 0.2 M) at 0 °C was treated successively with CBr_4 (2.5 g, 7.54 mmol, 1.3 equiv) and PPh_3 (2.15 g, 8.19 mmol, 1.4 equiv) and the reaction mixture was

stirred at 4 °C for 10 h. The reaction mixture was then concenctrated under reduced pressure and washed repeatedly with Et_2O (8 x 10 mL washes). The Et_2O washes were combined and concentrated under reduced pressure. Chromatography (SiO_2, 5 cm x 15 cm, hexanes) afforded the intermediate as a clear, colorless oil (1.02 g, 1.47 g theorectical, 69.5 %): ^{1}H NMR ($CDCl_3$, 250 MHz) d 3.64 (s, 3H, C(O)OCH_3), 3.38 (t, 2H, J = 6.8 Hz, CH_2Br), 2.29 (t, 2H, J = 7.4 Hz CH_2C(O)OCH_3), 1.83 (p, 2H, C$H_2$$CH_2$Br), 1.63 (m, 2H, C$H_2$$CH_2$C(O)$OCH_3$) 1.47-1.28 (m, 8H, alkyl protons).

21. 9-*T*-butyldiphenylsilyloxy-nonanal (intermediate for compound 100; Figure 5)

A solution of 1 (1.25 g, 2.93 mmol, 1.0 equiv) in toluene (9.80 mL, 3.0 M) at -78 °C was treated dropwise with DIBAL-H (4.40 mL, 1.0 M solution in hexanes, 4.40 mmol, 1.5 equiv). The reaction mixture was stirred at -78 °C for 30 min. The reaction mixture was then treated dropwise with MeOH (2 mL) and partitioned between EtOAc (100 mL) and H_2O (100 mL). The organic layer was washed with 10 % aqueous HCl (100 mL), dried (Na_2SO_4), and concentrated under reduced pressure. Chromatography (SiO_2, 5 cm x 15 cm, 0-5 % EtOAc-hexanes gradient elution) afforded 3 as a colorless oil (1.1 g, 94.9 %): ^{1}H NMR ($CDCl_3$, 250 MHz) δ 9.76 (t, 1H, J = 1.8 Hz, *H*C(O)R), 7.67 (m, 4H, ArH), 7.40 (m, 6H, ArH), 3.65 (t, 2H, J = 6.4 Hz, CH_2OTBDPS), 2.41 (t of d, 2H J = 1.8 and 7.3 Hz, CH_2C(O)H), 1.58 (m, 4H, C$H_2$$CH_2$OTBDPS and C$H_2$$CH_2$C(O)H), 1.29 (b s, 8H, alkyl protons), 1.05 (s, 9H, (CH_3)$_3$C); FABHRMS (NBA/CsI) *m/e* 529.1560 (C25H36O2Si + Cs^+ requires 529.1539).

22. <u>Methyl-9-triphenylphosphoranyl-nonanoate Bromide (intermediate for compound 100: Figure 5)</u>

A solution of 9-*T*-butyldiphenylsilyloxy-nonanal (1.02 g, 4.06 mmol, 1.0 equiv) in CH_3CN (3.5 mL, 1.16 M) was treated with triphenylphosphine (1.17 g, 4.47 mmol, 1.1 equiv) and stirred at reflux for 10 h. Additional triphenylphosphine (0.532 g, 2.03 mmol, 0.5 equiv) was added to the reaction mixture and stirring was continued at reflux for 5 h. The reaction mixture was concentrated under reduced pressure and washed repeatedly with Et_2O (5 x 10 mL washes). The remaining residue was then solubilized in the minimum volume of CH_2Cl_2 and concentrated under reduced pressure to afford the intermediate as a colorless foam (1.90 g, 2.08 g theoretical, 91.3%): ^1H NMR ($CDCl_3$, 250 MHz) δ 7.82-7.51 (m, 15H, ArH), 3.70-3.46 (m, 5H, $CH_3OC(O)R$ and CH_2PPh_3), 2.13 (t, 2H, J = 7.4 Hz, $CH_2C(O)OCH_3$), 1.62-1.02 (m, 12H, alkyl protons); FABHRMS (NBA) *m/e* 433.2312 ($C_{28}H_{34}BrO_2P$ - Br^- requires 433.2296).

23. <u>Methyl-18-t-butyldiphenysilyloxy-*cis*-9,10-octadecenoate (intermediate for compound 100: Figure 5)</u>

A solution of (1.0 g, 1.95 mmol, 1.0 equiv) in THF (6.5 mL, 0.3 M) at 25 °C was treated with KHMDS (3.9 mL, 0.5 M solution in THF, 1.95 mmol, 1.0 equiv) and the reaction mixture was stirred at reflux for 1 h. The reaction mixture was then cooled to -78 °C, treated with 3 (0.93 g, 2.35 mmol, 1.2 equiv), warmed to 25 °C, and stirred for an additional 30 min. The reaction mixture was then treated with saturated aqueous NH_4Cl and partitioned between EtOAc (100 mL) and H_2O (100 mL). The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. Chromatography (SiO_2, 5 cm x 15 cm, 0-2%

EtOAc-hexanes gradient elution) afforded the intermediate as a colorless oil (0.82 g, 1.07 g theoretical, 76.3%): ^1H NMR ($CDCl_3$, 250 MHz) δ 7.67 (m, 4H, ArH), 7.41 (m, 6H, ArH), 5.34 (m, 2H, *CH*=*CH*), 3.65 (m, 5H, $CH_3OC(O)$ and $CH_2OTBDPS$), 2.29 (t, 2H, *J* = 7.4 Hz, $CH_2C(O)OCH3$), 2.00 (m, 4H, $CH_2CH{=}CHCH_2$), 1.55 (m, 4H, $CH_2CH_2C(O)OCH_3$ and $CH_2CH_2OTBDPS$), 1.29 (b s, 18H, alkyl protons), 1.04 (s, 9H, $(CH_3)_3C$).

24. <u>18-*T*-butyldiphenylsilyloxy-*cis*-9,10-octadecenoic acid (compound 100: Figure 5)</u>

A solution of Methyl-18-t-butyldiphenysilyloxy-*cis*-9,10-octadecenoate (0.81 g, 1.47 mmol, 1.0 equiv) in THF-MeOH-H_2O (3-1-1 ratio, 7.3 mL, 0.2 M) at 0 °C was treated with $LiOH{\cdot}H_2O$ (0.188 g, 4.48 mmol, 3.0 equiv). The reaction mixture was warmed to 25 °C, stirred for 8 h, and then partitioned between EtOAc (100 mL) and H_2O (100 mL). The organic layer was washed successively with 10% aqueous HCl (100 mL) and saturated aqueous NaCl (100 mL), dried, and concentrated under reduced pressure. Chromatography (SiO_2, 5 cm x 15 cm, 10-30% EtOAc-hexanes gradient elution) afforded 100 as a colorless oil (0.700 g, 0.790 g theoretical, 88.7%): ^1H NMR ($CDCl_3$, 250 MHz) δ 7.67 (m, 4H, ArH), 7.41 (m, 6H, ArH), 5.34 (m, 2H, *CH*=*CH*), 3.65 (t, 3H, J = 6.5 Hz, $CH_2OTBDPS$), 2.34 (t, 2H, *J* = 7.4 Hz, CH_2COOH), 2.00 (m, 4H, $CH_2CH{=}CHCH_2$), 1.65-1.50 (m, 4H, CH_2CH_2COOH and $CH_2CH_2OTBDPS$), 1.47-1.23 (m, 18H, alkyl protons), 1.05 (s, 9H, $(CH_3)_3C$); FABHRMS (NBA/CsI) *m/e* 669.2772 ($C_{34}H_{52}O_3Si + Cs^+$ requires 669.2740).

25. <u>Synthesis of Compound 101 (Figure 5)</u>

Step 1. A solution of 100 (1.0 equiv) in CH_2Cl_2 (0.3 M) at 0 °C

was treated dropwise with oxalyl chloride (4.0 equiv). The reaction mixture was stirred at 25 °C for 4 h, concentrated under reduced pressure, cooled to 0 °C, and treated with saturated aqueous NH_4OH (2.0 mL). The reaction mixture was then partitioned between EtOAc (100 mL) and H_2O (100 mL), and the organic layer was dried (Na_2SO_4) and concentrated under reduced pressure.

Step 2. A solution of the above step 1 intermediate compound (1.0 equiv) in ether (0.3 M) at 0 °C was treated dropwise with pyridine (8.0 equiv.) followed by trifluoroaceticanhydride (6.0 equiv; Aldrich). The reaction mixture was stirred at 25 °C for 3 h, concentrated under reduced pressure, cooled to 0 °C, and treated with saturated aqueous NH_4OH (2.0 mL). The reaction mixture was then partitioned between EtOAc (100 mL) and H_2O (100 mL), and the organic layer was dried (Na_2SO_4) and concentrated under reduced pressure.

Step 3. A solution of the above step 2 intermediate compound (1.0 equiv) in THF (0.31 M) was treated with tetrabutylammoniumfluoride (1.0 M solution in THF, 3.0 equiv) and the reaction mixture was stirred at 25 °C for 3 h. The reaction mixture was then partitioned between EtOAc (50 mL) and H_2O (50 mL), and the organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. Product was purified by standard chromatographic conditions and yielded compound 101 in 66% overall yield for the 3 steps.

26. <u>Synthesis of Compound 102 (Figure 5)</u>

Step 1. A solution of 101 (1.0 equiv.) in THF (0.1 M) was

treated with triphenylphosphine (2.0 equiv.), followed by diethylazodicarboxylate solution (1.0 THF solution, DEAD, 2.0 equiv., Aldrich) and at 0 °C for 30 minutes. The reaction mixture was concentrated under reduced pressure and washed repeatedly with Et_2O (5 x 10 mL washes). The remaining residue was then solubilized in the minimum volume of CH_2Cl_2 and concentrated under reduced pressure.

Step 2. A solution of the above step 1 compound (1.0 equiv.) in THF (0.10 M) was treated with thiolacetic acid (2.0 equiv.; Aldrich) at 0 °C for 30 minutes. The reaction mixture was concentrated under reduced pressure and washed repeatedly with Et_2O (5 x 10 mL washes). The remaining residue was then solubilized in the minimum volume of CH_2Cl_2 and concentrated under reduced pressure. Product was purified by standard chromatographic conditions and yielded compound 102 in 71% overall yield for the 2 steps.

27. Synthesis of Compound 103 (Figures 4 & 5)

Step 1. A solution of 102 (1.0 equiv) in MeOH/Water (2:1 mixture, total concentration 0.20 M) at 0 °C was treated with NaOH (3.0 equiv) and stirred for 10 minutes, and then partitioned between EtOAc (100 mL) and water (100 mL). The organic layer was washed successively with 10% aqueous HCl (100 mL) and saturated aqueous NaCl (100 mL), dried, and concentrated under reduced pressure.

Step 2. A solution of the above step 1 compound (1.0 equiv) in aqueous 1N HCl at 0 °C was stirred until the reaction mixuture achieved a pH of 7.0, and then the mixture was partitioned

between EtOAc (100 mL) and water (100 mL). The organic layer was washed successively with saturated aqueous NaCl (100 mL), dried, and concentrated under reduced pressure.

Step 3. A solution of the above step 2 compound (1.0 equiv.) in aqueos 1mM $NaHCO_3$ at 25 °C was treated with Pyridyl disulfide beads (1.1 equiv. Aldrich) and stirred for 2 hours. The beads were subsequently washed with excess saturated $NaHCO_3$ (3X), water (3X) and brine (1X). Standard filtration obtained the activated beads (compound 103) which were then packed into the column for affinity chromatography of the enzyme as discussed supra using this CF3-inhibitor linked to activated pyridyl disulphide beads.

D. Cloning of *Cis*-9,10-Octadecenoamidase cDNA

1. *Cis*-9,10-Octadecenoamidase cDNA Obtained from Rat Liver mRNA

To obtain a cDNA clone for *cis*-9,10-octadecenoamidase from cDNA library generated from rat liver mRNA, degenerate oligonucleotide primers were designed based on the amino acid residue sequence of *cis*-9,10-octadecenoamidase polypeptide fragment obtained from a trypsin digest. Briefly, the *cis*-9,10-octadecenoamidase, purified as described above, was subjected to a trypsin digest to form internal polypeptide fragments as performed by Worchester Foundation, Worchester, PA. The resultant polypeptide fragments were purified by HPLC and seven HPLC fractions showing discrete peptide masses as measured by Matrix-Assisted-Laser-Desorption-Ionization with Time-of-Flight (MALDI TOF, PerSeptive Biosystems Linear Instrument) mass spectrometry were selected for microsequencing. Seven

polypeptide fragments were microsequenced having lengths ranging from 12 to 25 amino acid residues as indicated in Figure 9 indicated by seven discontinuous singly underlined regions in the complete rat *cis*-9,10-octadecenoamidase amino acid residue sequence. Each peptide possessed the required lysine or arginine residue at its C-terminus indicating that the tryptic digest proceeded with the anticipated selectivity.

The degenerate oligonucleotide primers were designed to incorporate a unique restriction site into the 5' ends of the primers that functioned as either forward and the backward primers. The forward primers are also referred to as upstream, sense or 5' primers. The backward primers are also referred to as downstream, anti-sense or 3' primers. The restriction sites were incorporated into the polymerase chain reaction (PCR) products to allow for insertion into the multiple cloning site of a sequencing vector as described below.

The synthesized 5' and 3' degenerate oligonucleotides were designed respectively corresponding to portions of sequenced peptides 1 and 2 as shown in Figure 9 as indicated by the first two discontinuous singly underlined amino acid residue sequences. The degenerate nucleotides are indicated by IUPAC codes N = A, C, G or T and R = A or G. The nucleotide sequence of the 5' degenerate primer corresponding to peptide 1 was 5'CGGAATTCGGNGGNGARGGNGC3' (SEQ ID NO 3) incorporating an EcoRI restriction site and translating into the amino acid sequence GGEGA (SEQ ID NO 4). The nucleotide sequence of the 3' degenerate primer that corresponded to peptide 2 was 5'CGGGATCCGGCATNGTRTARTTRTC3' (SEQ ID NO 33) incorporating an BamHI restriction site and translating into the amino acid sequence DNYTMP (SEQ ID NO 34).

To amplify regions of cDNA encoding *cis*-9,10-octadecenoamidase, rat liver mRNA was reversed transcribed into cDNA for use a template in PCR with selected pairs of degenerate oligonucleotide primers described above. PCR was performed under conditions well known to one of ordinary skill in the art with each cycle of 40 total cycles having the temperatures 94°C for 30 seconds, 60°C for 45 seconds and 72°C for 60 seconds.

Of the cloned PCR fragments, three were selected for sequencing. The three PCR fragments were 350 base pairs (bp), 400 bp and 750 bp. Sequencing of these *cis*-9,10-octadecenoamidase-encoding cDNA fragments showed that the 750 bp fragment contained the sequences of both the 350 and 400 bp fragments.

The 350 bp cDNA fragment obtained by PCR was then labeled internally and used as a probe for Northern analysis on electrophoresed rat liver mRNA. The probe hybridized to a fragment approximately 2.5 to 3.0 kilobases (kb) in length, which is the expected size of the *cis*-9,10-octadecenoamidase mRNA that encodes a 60 kDa protein.

To isolate a cDNA clone encoding the complete *cis*-9,10-octadecenoamidase protein, the 350 bp probe was then internally labeled with ^{32}P used to screen a λgt11 cDNA library from rat liver mRNA obtained from Clontech (Palo Alto, CA). For screening, the amplified 350 bp fragment was first digested with EcoRI and BamHI for directional cloning into a similarly digested pBluescript II SK(-)(Stratagene, La Jolla, CA). The resultant sequence indicated that the 350 bp fragment encoded the peptides 1 and 2 from which the degenerate oligonucleotide primers were designed confirming the accuracy of the PCR and amplification of the desired clone. The methods for cloning the

cis-9,10-octadecenoamidase cDNA of this invention are techniques well known to one of ordinary skill in the art and are described, for example, in "Current Protocols in Molecular Biology", eds. Ausebel et al., Wiley & Sons, Inc., New York (1989), the disclosures of which are hereby incorporated by reference.

Four positive clones were identified from a screening of 4.5 X 10^5 plaques. Two clones of 2.7 kb in length and 1 of 2.0 kb in length, were obtained. The partial sequence of one of the 2.7 kb clones, designated p60, indicates that the clone does contain *cis*-9,10-octadecenoamidase-specific sequences.

The rat liver cDNA clone designated p60 obtained above has been deposited with American Type Culture Collection (ATCC) on or before June 12, 1996 and has been assigned the ATCC accession number 97605. This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable plasmid for 30 years from the date of each deposit. The plasmid will be made available by ATCC under the terms of the Budapest Treaty which assures permanent and unrestricted availability of the progeny of the plasmid to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 U.S.C. §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638). The assignee of the present application has agreed that if the plasmid deposit should die or be lost or destroyed when cultivated under

suitable conditions, it will be promptly replaced on notification with a viable specimen of the same plasmid. Availability of the deposit is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

A partial nucleotide sequence of the top strand of the p60 cDNA clone containing 780 nucleotides described above is listed in SEQ ID NO 1 along with the deduced amino acid residue sequence. The encoded amino acid residue sequence is listed separately in SEQ ID NO 2. In order to show the amino acid residue encoded by each triplet codon in the Sequence Listing, a stop codon, TAA, was added at positions 781 to 783 to allow for the coding sequence (CDS) function in the Patentin program used to prepare the Sequence Listing. In other words, the stop codon is artificially inserted into the nucleotide sequence shown in SEQ ID NO 1 to facilitate the translation of the cDNA coding sequence into an amino acid sequence.

The actual position of the *cis*-9,10-octadecenoamidase nucleotide position within a complete cDNA clone is evident from the complete cDNA sequence as described below.

The two largest positive cDNA clones were then cloned into pBluescript II SK(+) and sequenced. One clone encoded a partially processed transcript containing the full coding sequence of the oleamide amidase with an additional 200 bp of intronic sequence. The other clone encoded a fully processed oleamide amidase transcript but fused to the 5' end of the clone was a 300 bp fragment encoding rRNA. Fusion of the two clones through an internal overlapping HindIII restriction site generated the full-length rat *cis*-9,10-octadecenoamidase also referred to as fatty acid amide hydrolase abbreviated as FAAH.